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(54) DETERMINATION OF POLYUNSATURATED FAT LEVELS IN BODY FLUIDS

(71) We, CHEMBRO HOLDINGS (PROPRIETARY) LIMITED, a company registered according to the laws of the Republic of South Africa, of 105 Quartz Street, Hillbrow, Johannesburg, Transvaal, Republic of South Africa, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates to a novel method for the measurement of polyunsaturated fat, i.e. fatty acid, levels in body fluids such as serum or plasma and to a reagent for use in this method.

The measurement of polyunsaturated fatty acid levels in body fluids, particularly serum and plasma, is a useful procedure in the clinical pathology laboratory or physician's rooms for monitoring the effects of diet and treatments on body fluid polyunsaturated fatty acid levels particularly in conditions associated with atherosclerosis and hypercholesterolaemia. The lengthy assay time of present established procedures for determining polyunsaturated fatty acid levels and the specialized equipment required prevents routine analysis on a wide scale at the present time.

Linoleic (9,12 octadecadienoic), linolenic (9,12,15 octadecatrienoic) and arachidonic (5,8,11,14 eicosatetraenoic) acids constitute the three main polyunsaturated fatty acids present in serum or plasma together with small amounts of pentaenoic and hexaenoic fatty acids. All of these acids contain the cis,cis-1,4-pentadiene system. Of these acids, linoleic acid is present in the greatest concentrations (usually up to 95%). Linoleic, linolenic and arachidonic acids are sometimes referred to as the essential fatty acids, that is, those fatty acids that cannot be biosynthesised or are synthesised in inadequate amounts by animals that require these nutrients for growth, maintenance and proper functioning of many physical processes. Polyunsaturated fatty acids constitute normally between 25 and 40% (w/v) of the total fatty acid content and are therefore present in normal subjects in the range 0.75 to 2.00 g/litre.

According to the invention, there is provided a method of determining the polyunsaturated fatty acid levels in a body fluid including the steps of converting the polyunsaturated fatty acid content of a sample of body fluid into free acid or salt form, taking a predetermined volume of this body fluid, oxidising the polyunsaturated fatty acids or salts in the volume of body fluid with molecular oxygen in the presence of excess of an oxygenase enzyme which is specific for polyunsaturated fatty acid which contain a cis,cis-1,4-pentadiene system in a suitable buffer, and measuring the amount of oxygen consumed by the volume of body fluid by means of an oxygen electrode.

An oxygen electrode is relatively inexpensive and enables the amount of oxygen consumed to be determined rapidly and in highly turbid or coloured solutions. The oxygen electrode is a polarographic device for measuring the concentration of oxygen dissolved in a given medium and depends on the electrolysis of dissolved oxygen at a weakly negative electrode. The oxygen electrode has been known since the early part of this century. In 1956, Clark improved the electrode considerably by using an oxygen permeable, non-conducting membrane to isolate the electrolytic cell from the sample under measurement—Clark, L. C., Trans. Am. Soc. Art. Int. Org. 2, 41, 1956. Oxygen electrodes are commercially available. The oxygen electrode can be coupled in known manner to a standard recorder such as a Cimatic Cimapot T5 Recorder for following and recording the rate and amount of oxygen consumption.

The amount of oxygen consumed by the

body fluid is directly proportional to the amount of polyunsaturated fatty acids in the body fluid. Since a sample of body fluid of predetermined volume is used the concentration of polyunsaturated fatty acids in the body fluid can be readily calculated. The oxygen electrode measures the amount of oxygen consumed and the amount of oxygen consumed is determined when equilibrium conditions are reached.

Sufficient molecular oxygen must, of course, be present to ensure that the content of polyunsaturated fatty acids or salts in the predetermined volume is oxidised. It is a simple matter to ensure that sufficient molecular oxygen is present because the likely concentrations of polyunsaturated fatty acids present in body fluids is known. The source of molecular oxygen is usually air saturated solutions.

The time it takes for equilibrium conditions to be reached is a function of the activity of the enzyme present. The greater the activity the quicker will the equilibrium conditions be reached. In all cases, however, there must be an excess of enzyme, i.e. sufficient enzyme activity present to catalyse the reaction and overcome any inhibiting effect of monounsaturated and saturated fatty acids present in body fluids. The amount of enzyme necessary is determinable without difficulty because the likely concentrations of fatty acids in body fluids are known.

Polyunsaturated fatty acids are present in body fluids in the form of esters. It is necessary to convert the esters into the free acid or salt form, prior to oxidation. It is preferred that the esters be converted into the salt form and this can conveniently be achieved by means of saponification. Saponification, as is known in the art, involves reacting an ester, usually with heat, with aqueous alkali, e.g. sodium or potassium hydroxide, to form an alcohol and the salt of the acid corresponding to the ester. Saponification is preferably achieved by means of a methanolic potassium hydroxide solution. It is a surpising aspect and an advantage of the invention that the oxidation can be performed on the salts of the acids.

If desired, the esters can be converted into free acid form. This is conveniently achieved by means of saponification followed by acidification, e.g. with a mineral acid such as hydrochloric acid, or by enzymic hydrolysis using for example lipases, cholesterol esterases or phospholipases. By using selected enzymes it is possible to determine the levels of polyunsaturated fatty acids esterified to cholesterol, glycerol or phospholipids. The oxygenase enzyme is preferably lipoxygenase (Linoleate: oxygen oxidoreductase E.C. No. 1.13.11.12).

The preferred buffer is one having a pH of 7 to 10. A particularly suitable buffer is a borate buffer of molarity 0.1 to 2.0 preferably 1.0, and a pH in the above range, preferably 9. The invention includes within its scope an oxygenase enzyme specific for polyunsaturated fatty acids which contain a cis,cis-1,4-pentadiene system in a preferred buffer as defined above.

The oxidation will generally take place at a temperature of 15 to 40°C. The method of the invention has a number of advantages over known methods of determining polyunsaturated fatty acid levels in body fluids such as the gas chromatographic method. The method of the invention is very rapid and utilises very small quantities of body fluids. Furthermore, as is mentioned above, it is not necessary to convert the esters of the body fluids into the free acids as the salts may be used.

An example of the invention will now be described. The following reagents were used:

Soya Bean Lipoxygenase:

This was purchased from Miles-Seravac, Cape Town, with an activity of 50,000 units/mg. One unit is defined by the manufacturers as the amount of enzyme which causes an increase in absorbance at 234 nm, due to the oxidation of linoleic acid, of 0.001 per minute at 25°C. 50,000 Miles-Seravac units=6 International Units at 25°C. This value was in fact obtained on assaying the lipoxygenase in the oxygen electrode with linoleic acid as substrate. Enzyme solutions were prepared by dissolving approximately 50 mg lipoxygenase in 1.0 ml of 1.0 M potassium borate buffer, pH 9.0.

Linoleic acid solution:

A standard solution was made by dissolving 80 μl (72 mg) linoleic acid in 10 ml absolute ethanol (25.8 micromoles/ml).

Buffer System:

1.0 M potassium borate buffer, pH 9.0 was used as the buffer for all experiments. This was prepared by dissolving 61.83 g crystalline boric acid in 500 ml distilled water and adding 20% (w/v) aqueous KOH to bring the pH to 9.0. The volume of this solution was then made to 1 litre by further addition of distilled water.

Methanolic-KOH:

14.3 g of potassium hydroxide were dissolved in 100 ml of methanol.

The oxygen consumption was measured using a commercially available oxygen electrode connected to a circulating water

bath. The electrode was covered by an 0.0005 inch Teflon membrane ("Teflon" is a Registered Trade Mark), and the cell volume was maintained at 1.5 ml. The output signal was recorded by means of a Cimatic Cimapot T5 recorder. The recorder was calibrated using air saturated water. The oxygen concentrations in air saturated solutions were calculated by the method of Glasstone (Glasstone S, Elements of Physical Chemistry, 1st Ed. pp 343—344, 1946, D. van Nostrand Co. Inc. New York).

In order to confirm that one molecule of oxygen is consumed per molecule of polyunsaturated fatty acid, and experiment was carried out to record the oxygen consumption obtained on addition of varying amounts of linoleic acid to a solution containing lipoxygenase. Linoleic acid was chosen because it is the major constituent of polyunsaturated fatty acids in body fluids.

1.5 ml of the potassium borate buffer were added to the oxygen electrode cell together with varying volumes (1 to 6 μl) of the standard linoleic acid solution. After thermal equilibration of the mixture at 37°C, 100 μl of lipoxygenase solution were added to start the reaction. Equilibrium conditions were reached after two or three minutes, and the amount of oxygen consumed after five minutes was plotted against the amount of linoleic acid (μmoles) added. The results are shown in the attached graph from which it can be seen that the stoichiometry of the reaction is 1:1 In the graph the amount of linoleic acid added in μmoles is plotted along the abscissa and the total oxygen consumed in μmoles is plotted along the ordinate.

The method of the invention was then carried out on serum and plasma. 50 μl of plasma or serum were pipetted into a small test-tube, 0.12 ml of the methanolic-KOH solution added and the tube covered and placed in a water-bath at 60°C for 10 minutes. The contents of the tubes after cooling were made up to 0.25 ml with methanol. 25 μl of the resulting solution were then added to 1.5 ml of the borate buffer in the reaction-chamber of the oxygen electrode. After temperature equilibration had been achieved at 37°C, the reaction was initiated by addition of 100 μl of the lipoxygenase solution (~5 mg). Equilibrium conditions were attained within 5 minutes, and the total oxygen consumed after 5 minutes was calculated from the recorder reading. Because of the direct relationship between the oxygen consumed and content of polyunsaturated fatty acids in the serum and plasma this reading gave the number of micromoles of polyunsaturated fatty acids present in the sample. Since the volume of the sample is known, the concentration of polyunsaturated fatty acids in the serum and plasma can be readily calculated.

WHAT WE CLAIM IS:—

1. A method of determining the polyunsaturated fatty acid levels in a body fluid including the steps of converting the polyunsaturated fatty acid content of a sample of body fluid into free acid or salt form, taking a predetermined volume of this body fluid, oxidising the polyunsaturated fatty acids or salts in the volume of body fluid with molecular oxygen in the presence of excess of an oxygenase enzyme which is specific for polyunsaturated fatty acids which contain a cis,cis-1,4-pentadiene system in a suitable buffer, and measuring the amount of oxygen consumed by the volume of body fluid by means of an oxygen electrode.

2. A method according to claim 1 wherein the body fluid is serum or plasma.

3. A method according to claim 1 or claim 2 wherein the oxygenase enzyme is lipoxygenase.

4. A method according to any one of the preceding claims wherein the buffer is one having a pH of 7 to 10.

5. A method according to any one of the preceding claims wherein the buffer is a borate buffer having a molarity of 0.1 to 2 molar.

6. A method according to any one of claims 1 to 3 wherein the buffer is a borate buffer having a molarity of 1.0 and a pH of 9.

7. A method according to any one of the preceding claims wherein the polyunsaturated fatty acid content of the body fluid is converted into salt form by saponification.

8. A method according to claim 7 wherein the saponification is achieved by means of a methanolic potassium hydroxide solution.

9. A method according to claim 1 substantially as herein described.

10. A reagent for use in the method of any one of the preceding claims comprising a borate buffer of molarity 0.1 to 2 molar and pH 7 to 10 and containing an oxygenase enzyme which is specific for polyunsaturated fatty acids containing a cis,cis-1,4-pentadiene system.

11. A reagent according to claim 10 having a molarity of 1.0 and a pH of 9.

12. A reagent according to claim 10 or 11 wherein the oxygenase enzyme is lipoxygenase.

13. A reagent according to claim 10 substantially as herein described.

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1 SHEET *This drawing is a reproduction of the Original on a reduced scale*